Role of nitric oxide in immune-mediated diseases

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INTRODUCTION

The demonstration in 1988 [1] that nitric oxide (NO) is a major mediator of cytotoxicity in activated macrophages has led to extensive research into its role in inflammation and as an effector molecule in immune diseases. It has also become apparent that NO production both by macrophages and other cells, including T-cells, may play a role in modulating the generation of the immune response. In this review we discuss the evidence for these actions of NO in immune-mediated diseases.

NO is a free radical synthesized by the oxidation of one of the terminal guanido-nitrogen atoms of the amino acid L-arginine by specific enzymes called nitric oxide synthases (NOSs) (for reviews see [2, 3]). In mammals, NOSs exist in three isoforms which differ in the way their activity is controlled [3]. All three isoforms depend for their activity on the binding of calmodulin, but in one isoform (NOS I) calmodulin is tightly bound at resting calcium concentrations, and therefore once the enzyme is synthesized it is active and controlled at the level of transcription and translation. This is the isoform that was originally shown to be inducible in murine macrophages by pro-inflammatory cytokines or lipopolysaccharide (LPS) and has subsequently been shown to be induced by similar stimuli in many other cell types. It is generally known as inducible NOS (iNOS). The other two isoforms are constitutively present in cells and are activated by transient rises in intracellular calcium. One of these constitutive enzymes (NOS I, cNOS) was first described in endothelium and the other (NOS III, nNOS) in neurons; they are responsible for the role of NO as an endothelium-derived vasodilator and as a neurotransmitter.

The stimuli which lead to synthesis of iNOS vary with cell type and species and in some cases a cocktail of cytokines is needed for induction. In rodent macrophages, interferon-γ (IFN-γ) is a potent inducer; tumour necrosis factor-α (TNF-α), interleukin (IL)-1 and LPS potentiate this effect. Several agents are able to suppress iNOS induction including glucocorticoids, macrophage deactivating factor, isoforms of transforming growth factor-β, platelet-derived growth factor, epidermal growth factor and IL-4 and IL-10. iNOS is also present in human cells. The gene has been localized to chromosome 17 [4] and the presence of iNOS has been demonstrated in human hepatocytes [5], chondrocytes [6], colon carcinoma cells [7] and renal mesangial cells [8]. It is not clear whether iNOS is present in human endothelial cells. In human umbilical vein endothelial cells cytokines stimulate NO synthesis [9], but this is via a calcium-dependent form of NOS and is mediated by increased tetrahydrobiopterin levels. In contrast, a calcium-independent NOS activity was induced by cytokines in a human endothelial cell line [10]. There is, as yet, no definitive molecular biological evidence for iNOS expression in human endothelial cells. It has proved much more difficult to induce iNOS in human macrophages than in rodent macrophages. In some cases iNOS mRNA and protein have been detected in human monocytes treated with IFN-γ and LPS without the generation of measurable NO or its metabolites [11], leading to speculation that these cells lack an essential cofactor such as tetrahydrobiopterin. However, it is now clear that NO production via iNOS in human mononuclear phagocytes does occur under certain tightly controlled conditions (reviewed in [12]). Human monocytes have been shown to produce significant levels of NO when infected with HIV, after cross-linking of the CD69 antigen, when stimulated by certain tumour cells or, under some circumstances, by IL-4. The effect of IL-4 appears to be mediated via CD23, a low-affinity IgE receptor, and this pathway has been shown to be a mechanism of NO-mediated killing.

Key words: immune complex diseases, immunity, inflammation mediators, nitric oxide, nitric oxide synthase.

Abbreviations: EAE, experimental allergic encephalomyelitis; IFN-γ, interferon-γ; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; L-NAME, Nω-nitro-L-arginine methyl ester; L-NMMA, Nω-monomethyl-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; RT-PCR, reverse transcriptase-polymerase chain reaction; Th1 cells, T-helper 1 cells; Th2 cells, T-helper 2 cells; TNF-α, tumour necrosis factor-α.

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of *Leishmania major* in human macrophages. This action of IL-4 is in contrast to that in rodent cells where, as noted above, its effect is to inhibit iNOS induction. The difficulty in demonstrating iNOS in human mononuclear phagocytes compared with those from rodents suggests that, in humans, iNOS may have significantly different roles and indicates that it is necessary to be cautious in extrapolating from rodent experiments.

NO has multiple effects on cells which reflects the wide range of targets to which it may bind [2, 13] (Table 1). It has a high affinity for metalloproteins and binds strongly to iron in haem groups or iron–sulphur groups. Binding to the haem in soluble guanylate cyclase activates the enzyme, leading to the generation of cGMP which is responsible for the activity of NO as a vasodilator and inhibitor of platelet aggregation. The cytotoxic effects of NO appear to be due to binding to iron-sulphur groups in enzymes of the mitochondrial respiratory transport chain, and to inhibition of DNA synthesis. At sites of inflammation NO may react with superoxide to form peroxynitrite [14]. The effect of this interaction *in vivo* is uncertain; it may have beneficial effects by scavenging superoxide or may enhance tissue damage by the subsequent formation of the highly reactive hydroxyl radical. It is likely that the balance of these effects depends on the relative concentrations of superoxide and NO, and on the levels of other antioxidants. NO may also affect the transcription and expression of other inflammatory mediators. In some circumstances NO causes feedback inhibition of cytokines which stimulate its synthesis such as TNF-α [15] although, at least *in vitro* and in certain cell types, NO has been shown to stimulate transcription of iNOS itself in a positive-feedback loop [16]. At inflammatory sites NO enhances the activity of cyclo-oxygenase which will promote the formation of vasodilator prostaglandins [17]. In several models NO has been shown to reduce the expression of endothelial adhesion molecules, thus reducing leucocyte infiltration in inflammation [18, 19].

There are three main ways in which NO synthesis has been investigated in immune-mediated diseases. First by demonstration of the synthesis of NO itself or of its metabolites, particularly the stable metabolites nitrite and nitrate; secondly by showing the upregulation of iNOS by examining its biochemical activity or demonstrating the presence of the protein or mRNA; thirdly by examining the effects of inhibitors of NOS *in vitro* or *in vivo*. The main inhibitors used are analogues of L-arginine such as N^ω^-monomethyl-L-arginine (L-NMMA). These experiments using inhibitors are hampered by the lack of selective inhibitors for iNOS and therefore *in vivo* interpretation is complicated by possible effects on endothelial NOS and thus on blood flow and systemic blood pressure.

### Table 1. Targets and actions of nitric oxide

<table>
<thead>
<tr>
<th>Target</th>
<th>Actions</th>
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<tbody>
<tr>
<td>Cytosolic</td>
<td>Increased cGMP. Vasodilatation. Inhibition of platelet aggregation.</td>
</tr>
<tr>
<td></td>
<td>Neurotransmission</td>
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<td></td>
<td>Scavenging of NO</td>
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<td>Transport of NO to microcirculation</td>
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<td>Haemoglobin</td>
<td>Inhibition of enzyme activity</td>
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<td>Iron-sulphur proteins</td>
<td>Cytoxicity</td>
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<tr>
<td>Aconitase</td>
<td></td>
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<tr>
<td>NADH: ubiquinone oxidoreductase</td>
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<tr>
<td>NADH: succinate oxidoreductase</td>
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<tr>
<td>Ribonucleotide reductase</td>
<td>Inhibition of DNA synthesis</td>
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<tr>
<td>Cycle-oxygenase</td>
<td>Increased activity</td>
</tr>
<tr>
<td>NOS</td>
<td>Inhibition</td>
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<tr>
<td>Superoxide anion</td>
<td>Scavenging of superoxide</td>
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<tr>
<td>Nuclear</td>
<td>Formation of peroxynitrite</td>
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<tr>
<td>Nuclear factor-κB</td>
<td>Activation</td>
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<tr>
<td>activator protein-1</td>
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<tr>
<td>DNA</td>
<td>Mutagenesis</td>
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<tr>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td>Extracellular</td>
<td>Transport and prolongation of action of NO</td>
</tr>
<tr>
<td>Albumin</td>
<td>Activation</td>
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<td>Plasminogen activator</td>
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**NO IN THE GENERATION OF THE IMMUNE RESPONSE**

The generation of the immune response involves the presentation to CD4+ T-helper cells of antigen by specialized antigen-presenting cells which express class II histocompatibility locus antigen. NO has been shown to inhibit class II histocompatibility locus antigen expression and so may interfere with antigen presentation [20]. In recent years it has become clear that there are two functionally distinct types of CD4+ T-helper cells. The T-helper 1 (Th1) subset synthesizes and secretes IL-2 and IFN-γ whereas Th2 cells produce IL-4, IL-5 and IL-10. These patterns of cytokine production largely determine the effector functions of the two subsets of T-cells. Thus, Th1 cells mediate delayed type hyper-
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Fig. 1. Involvement of NO in the modulation of the immune response. NO derived from accessory cells and T-lymphocytes inhibits T-cell proliferation and MHC class II expression. Differential cytokine production by T-helper subsets regulates the induction of iNOS. Th0, uncommitted T-cell; Th, T-helper cell; CTL, cytototoxic T-cell; Mac, macrophage.

sensitivity and, through IFN-γ secretion, will activate macrophages and stimulate NO production. IL-4 secreted by Th2 cells is of critical importance for IgE production and leads to expression of vascular cell adhesion molecule 1 which is required for adhesion of eosinophils. The Th2 cytokines IL-4 and IL-10 are known to downregulate iNOS, and IL-10 also inhibits the secretion of IFN-γ by Th1 cells. Thus the balance between Th1 and Th2 cells may determine the outcome of infectious and autoimmune diseases and experimental evidence suggests that NO may be involved in the control of this balance.

Figure 1 shows a schematic diagram of the ways in which NO may be involved in modulation of the immune response. The first evidence of a role for NO in the generation of the immune response came from Hoffman et al. [21] who showed that nitrite and nitrate are produced in the mixed lymphocyte response of mouse spleen cells and that inhibition of NOS with the competitive inhibitor L-NMMA markedly increased the proliferative response. On the basis of these and other experiments [22] it has been suggested that IFN-γ produced by activated Th1 cells induces iNOS synthesis in macrophages, causing NO synthesis which then inhibits proliferation of cytokotoxic T-lymphocytes. There is also evidence in vivo that NO may inhibit proliferation of T-cells. In experimental trypanosomiasis, NO production by activated macrophages inhibits T-cell proliferation [23] and treatment of infected mice with L-NMMA leads to a significant reduction in parasitemia [24]. A similar phenomenon is seen with Listeria infection [25] if NO production is inhibited by a single dose of L-NMMA at the time of infection. However, if NO synthesis is inhibited throughout the course of infection with Listeria the bacterial load is increased, suggesting that the relative effects of NO on micro-organisms and on T-cells may change with time [26].

In addition to activated macrophages, lymphocytes themselves are also a possible source of NO. In the mouse, Th1 cells can be activated to produce large amounts of NO which can inhibit the secretion of IL-2 and IFN-γ by Th1 cells, thus acting as an autoregulator of the immune response [27] and possibly altering the balance of Th1 and Th2 cells. However, others have been unable to demonstrate T-cell synthesis of NO [28]. B-cells may also be a source of NO, and in humans it has been reported that Epstein-Barr virus-transformed B-cells and cell lines from Burkitt's lymphoma express iNOS [29]. There may be strain differences in the generation of NO which explain the tendency to develop a particular type of immune response. Thus, in the Brown Norway strain of rat, administration of mercuric chloride causes a systemic autoimmune syndrome of Th2 type characterized by a dramatic increase in serum IgE levels and production of autoantibodies to the glomerular basement membrane. In spleen cell cultures from Brown Norway rats exposed to mercuric chloride the level of NO is significantly enhanced compared with control Lewis rats, resulting in a suppression of IFN-γ synthesis which may contribute to the development of the Th2 response [30].

Evidence of a role for NO in generation of the immune response is also seen in mice homozygous for a targeted disruption of the iNOS gene [31, 32]. These knockout mice are highly susceptible to Listeria [32] and Leishmania infection [31]. Spleen cells from knock-out mice showed higher levels of T-cell proliferation than those from wild-type mice when cultured with leishmanial antigens or concanavalin A, suggesting a role for NO in inhibiting T-cell proliferation. When stimulated with soluble leishmanial antigens, spleen cells produced more IFN-γ but less IL-4 than those from wild-type mice, consistent with the hypothesis that NO may prevent the overexpansion of Th1 cells relative to Th2 cells [31].

IMMUNE DISEASES

There is now considerable evidence suggesting that NO is involved in the pathogenesis of immunologically mediated diseases [33]. In this section, the evidence for NO synthesis in vivo in experimental models and human immune diseases, and the effects of NO inhibition on immunopathology are reviewed.

Immune complex diseases

Immune complex formation generates a wide range of cellular and molecular inflammatory mediators. The involvement of macrophages and cytokines suggests that NO could also be a mediator in these reactions. The diseases of immune
Glomerulonephritis. The evidence for NO synthesis in glomerulonephritis is now considerable, although its role in pathogenesis is far from resolved. The first evidence of a role for NO was in rat nephrotoxic nephritis, a model of immune complex injury by immune complex deposition, and vasculitis. Glomerular infiltrating the glomerulus, and depletion of nitrite by nephritic glomeruli has since been found in three other models of glomerulonephritis with features resembling acute post-infectious glomerulonephritis [35], membranous glomerulonephritis [36] and mesangial proliferative glomerulonephritis [37]. In all these models the quantity of nitrite generated is closely related to the numbers of macrophages infiltrating the glomerulus, and depletion experiments with irradiation showed that macrophages were the major source of glomerular nitrite [36], although intrinsic cells, particularly mesangial cells [38], may also contribute. The presence of iNOS mRNA [39, 40] and of iNOS protein, detected immunohistochemically [41], in nephritic glomeruli has confirmed that NO synthesis in glomerulonephritis is due to the induction of the inducible isofom of NOS. In a model of mesangial proliferative glomerulonephritis induced in the rat by antibody against the Thy 1 antigen on mesangial cells, there is an increase in glomerular mRNA for iNOS by 1 h (Fig. 2). In rat nephrotoxic nephritis the increase in iNOS mRNA persists until at least day 7 [39].

A number of studies have addressed the role of induced NO in glomerulonephritis in vivo, but interpretation is complicated as currently available NO inhibitors may also inhibit constitutive NO, leading to changes in both systemic and glomerular haemodynamics [42, 43] with possible exacerbation of immune complex injury by hypertension [44]. The first in vivo evidence of a role for NO in the pathogenesis of glomerulonephritis came from a study of chronic NO inhibition in spontaneous murine autoimmune disease [45] in MRL-lpr/lpr mice which spontaneously develop an autoimmune disease characterized by autoantibody production, glomerulonephritis, vasculitis and arthritis. These mice have evidence of increased iNOS activity with increased urinary nitrite/nitrate and large numbers of iNOS-positive cells in the kidney and spleen, iNOS mRNA is present. Treatment with oral L-NMMA, beginning at 8 weeks of age, reduced NO production, reduced proteinuria and ameliorated the histological changes of proliferative glomerulonephritis and arthritis. These results strongly implicate NO in this autoimmune disease. L-NMMA treatment did not reduce serum anti-DNA antibodies, suggesting that NO was mainly involved in the effector phase rather than in the generation of the immune response.

In the rat Thy 1 model of mesangial proliferative glomerulonephritis, a single dose of L-NMMA given 1 h before induction of disease almost abolished mesangiolysis and significantly reduced proteinuria and the later increases in mesangial matrix, suggesting that NO is a major effector of mesangial injury in this model [46].

In contrast with these results, others have found a protective role for NO in glomerulonephritis. In the heterologous phase of rat nephrotoxic nephritis L-NMMA infusion caused a marked increase in urinary protein excretion, accompanied by further increases in the elevated glomerular pressure and efferent arteriolar resistance seen in this model [47]. This suggests a role for induced NO in counteracting vasoconstriction in acute nephrotoxic injury. Preliminary experiments by the same group using the NOS inhibitor N^N^'-nitro-L-arginine methyl ester (L-NAME) and a milder level of injury also showed an exacerbation of proteinuria, and an increase in neutrophil infiltration, again suggesting a protective role for NO [48]. However, in both these experiments there were significant increases in mean arterial blood pressure. Similarly, experiments with L-NAME in passive [49] and active [50] Heymann nephritis which resulted in exacerbation of proteinuria, have been complicated by hypertension. There is a preliminary report on the use of aminoguanidine, a relatively selective iNOS inhibitor, in autoimmune glomerulonephritis induced by mercury chloride [51]. Aminoguanidine profoundly enhanced proteinuria when administered during the effector phase of the disease, but was not effective in the induction phase, again suggesting a protective role for NO in glomerular immune injury.

In an attempt to avoid the effects of competitive NO inhibitors on constitutive NO synthesis, we...
have recently performed studies to determine whether a more selective form of NO inhibition can be achieved by acute arginine depletion, as the high output of NO resulting from the inducible isofrom of NOS, particularly in macrophages, is dependent on L-arginine concentration. Arginase is a urea cycle enzyme which catalyses the metabolism of L-arginine to urea and L-ornithine. Exogenous arginase can inhibit NO production in vitro in both macrophages [52], and nephritic glomeruli [53]. Injection of bovine liver arginase in rats causes rapid depletion of plasma L-arginine levels without an increase in systemic blood pressure, suggesting that the function of endothelial NOS is not affected. In accelerated nephrotic nephritis this depletion of plasma arginine was accompanied by exacerbation of proteinuria and reduced glomerular nitrite synthesis [53]. These results also suggest a protective role for NO in immune complex glomerulonephritis. The mechanism by which NO exerts its protective effect is not clear. It may be that NO generation has a beneficial effect on glomerular haemodynamics. Another possibility is that inhibition of NO leads to the unopposed activity of superoxide. A third possibility is that NO acts to limit leucocyte infiltration either by inhibiting adhesion molecule [18, 19] or chemokine expression. There is a preliminary report that in rats given LPS, inhibition of NO leads to an increase in glomerular expression of the chemokine RANTES [54]. There is, as yet, no demonstration of NO synthesis in human glomerulonephritis.

**Lung and skin reactions.** Evidence from models of Arthus-type lung and vascular injury also provides data suggesting a significant role for NO in immune complex injury. The intratracheal instillation of IgG anti-BSA antibody followed by intravenous injection of BSA results in acute pulmonary inflammation with increased vascular permeability, haemorrhage, neutrophil infiltration and nitrate generation; the vascular changes are abrogated by co-instillation of the competitive NO inhibitor L-NAME [55]. The protective effect of L-NMMA is reversed when L-arginine is added to the intratracheal instillate. The anti-BSA antibody class determines the character of the leucocyte infiltrate in this model. If IgA antibody is used to induce disease, more than 90% of infiltrating cells are macrophages [56]. NO inhibition in this situation not only inhibits vascular changes, it also markedly decreases the macrophage infiltrate, in contrast to the lack of effect on neutrophil infiltration in the previous experiment. These two experiments point to a role for NO in both tissue injury and in macrophage recruitment.

Dermal vasculitis induced by BSA-anti-BSA Arthus reaction is also inhibited by L-NMMA [57], without a reduction in neutrophil infiltration. As neutrophils are known to be essential to this form of injury, the results suggest that NO release is a mechanism whereby neutrophils cause tissue damage (although there is some controversy concerning the ability of neutrophils to produce NO). As neutrophil-mediated injury is partly oxygen-radical dependent, the damaging effect of NO may be through peroxynitrite formation. Competitive NO inhibition with L-NAME also ameliorates skin Arthus reactions in the guinea pig, a species where neutrophils may be less important as effector cells [58]. Arthus reactions can also be induced in the peritoneal cavity by the intraperitoneal injection of specific antibody followed by intravenous antigen. Adherent peritoneal cells from these lesions have enhanced ex vivo nitrite production and calcium-independent NOS activity, and express iNOS mRNA [59]. However, NO inhibition in these experiments did not prevent increases in permeability, in contrast to the results in experimental immune complex lung injury. NO inhibition has been shown to attenuate histological tissue injury in a model of autoimmune necrotizing vasculitis induced by the polyclonal B-cell activator mercer chloride [60].

NO has also been implicated in allergic lung diseases characterized by mast cell and eosinophil infiltration such as asthma (reviewed in [61]). NO is continuously produced in the airways with significant levels in exhaled air [62]. Expression of iNOS has been found in vivo in normal bronchial epithelium at all levels of the bronchial tree [63], although in vitro bronchial epithelium requires stimulation by cytokines for iNOS induction [64]. In asthmatic patients the peak exhaled NO concentration is higher than in non-asthmatic subjects [62], and levels are reduced by inhaled steroid therapy [62]. Increased levels of NOS activity have also been found in lung samples from patients with inflammatory lung disease including asthma and cystic fibrosis [65]. The source of NO and the role of increased NO synthesis in asthma is still uncertain. In addition to the upregulation of NO synthesis in bronchial epithelium, there may also be a contribution from inflammatory cells. Histamine has also been shown to stimulate NO release from vascular endothelium via H_1_ receptors. The effects of NO may be multiple. It is both a bronchodilator and a pulmonary vasodilator. In larger amounts it may have cytotoxic effects or promote bronchial oedema and inflammation. In an animal model, anaphylactic hypotension mediated by mast cell histamine can be inhibited by pretreatment with L-NAME [66], suggesting that the vasodilatory effect of histamine is at least partly achieved through NO release.

**Cell-mediated immune diseases**

**Autoimmune diabetes.** The diabetes-prone BB rat strain, and the non-obese diabetic mouse represent spontaneous models of human type I diabetes with autoimmune T-cell-dependent pancreatic destruction. The pancreatic islets exhibit a chronic inflammatory reaction with infiltrating macrophages and
CD4+ cells. There are now many in vitro studies showing that cytokine-stimulated islet cells produce NO; furthermore, NO secretion by activated macrophages can destroy islet cells [67]. Culture of islets in vitro has shown that NO inhibits insulin release by reducing the oxidative capacity of the islet cell. In vivo, iNOS mRNA is expressed in the pancreatic lesions, and immunohistochemistry shows localization of iNOS in areas of macrophage infiltration [68]. Treatment of non-obese diabetic mice with aminoguanidine has reduced NO production in isolated islets and delayed the development of diabetes [69]. These studies provide evidence of a pathogenic role for NO. There are no in vivo data for human diabetes, there are conflicting reports on the sensitivity of human islet cells to NO [70-72], and aminoguanidine does not appear to be protective for human cells [72].

**Arthritis.** A chronic immune arthritis can be induced in rats by intraperitoneal injection of peptidoglycan-polysaccharide fragments derived from group A streptococcal cell walls. The joint lesions are similar to human rheumatoid arthritis, with mononuclear cell infiltration of synovium, cartilage degradation and bone erosion. Nitrite generation has been demonstrated ex vivo in the inflamed synovium excised from the joints, and iNOS mRNA and enzymic activity is present [73]. Treatment with L-NMMA virtually eliminates the joint swelling and reduces the cellular infiltration [73], showing that NO production is involved in this immunologically mediated arthritis. Similar results have been obtained in models of adjuvant arthritis induced by injection of heat-killed mycobacteria [74]. The role of NO in human rheumatoid arthritis has also been studied by examining the production of nitrite by synovial fluid [75]. The nitrite concentration was higher in patients with active disease than in those with quiescent disease or osteoarthritis. Immunohistochemistry and in situ hybridization have shown that iNOS is expressed in inflamed rheumatoid synovium by macrophages, synoviocytes, chondrocytes and endothelial cells [76]. Nitrite is also elevated in serum [75] and urine [77], and is reduced by prednisolone treatment [77].

Nitrotyrosine has been detected in the serum and synovial fluid of patients with rheumatoid arthritis, suggesting that there has been peroxynitrite formation [78].

**Experimental allergic encephalomyelitis.** Experimental allergic encephalomyelitis (EAE) is an inflammatory central nervous system demyelinating disease induced by immunization with myelin components, which is studied as a model of human multiple sclerosis. Although the pathogenic mechanisms underlying the damage to myelin have not been defined, a predominantly cell-mediated reaction is implicated, as disease can be transferred by sensitized T-lymphocytes, and mononuclear cell infiltration around cerebral blood vessels is a prominent histological feature. Leucocytes isolated from the central nervous system have increased synthesis of nitrite and nitrate, which is enhanced by in vitro co-incubation with encephalitogenic T-cell-lines activated by myelin basic protein [79], and iNOS mRNA is present in EAE brain [80]. NO has been localized in the spinal cords of mice with EAE by EPR spectroscopy [81]. An in vivo role for this induced NO synthesis is supported by the ameliorative effects of aminoguanidine treatment which both delayed onset and reduced inflammation and demyelination [82]. Excessive NO production could be acting as a cytotoxic agent in this disease, injuring glial cells and thereby reducing myelin production. In humans, Northern blots and reverse transcriptase–polymerase chain reaction (RT–PCR) analysis have shown that iNOS is present in the brains of those dying of multiple sclerosis but not in normal control subjects [83]. iNOS mRNA was localized to macrophages using RT–in situ PCR.

**Intestinal disease.** Intestinal graft-versus-host disease, induced by intraperitoneal injection of parental spleen cells into F1 hybrids, is a mouse model of immune intestinal diseases such as coeliac disease. The small intestine shows increased enterocyte proliferation, associated with increases in crypt length, and intraepithelial lymphocytes. The spleen is enlarged. The pathogenesis of this allo-specific immune reaction involves activated natural killer cells. Treatment with L-NMMA abolished gut injury and inhibited enhanced natural killer activity, indicating that NO is involved in the immunopathology of intestinal graft-versus-host disease [84]. The source of NO and its role in pathogenesis have not yet been clarified. It may mediate activation of natural killer cells. Human ulcerative colitis is of unknown aetiology, but an immune basis is suspected. Acute ulcerative colitis is associated with increased calcium-independent NOS activity in the mucosa [85] and increased concentrations of NO in luminal gas, particularly in parts of the colon with the most severe inflammation [86]. It is not clear whether this increased NO production is involved in pathogenesis, and if so whether its effects are toxic or beneficial. No effect on concentrations was detected with steroid therapy.

**Granulomas.** Granulomas are focal collections of mononuclear leucocytes, predominantly macrophages, which arise in response to persistent irritants. In hypersensitivity, a cell-mediated immune reaction involving T-cell chemokines and cytokines is central to the recruitment and activation of macrophages. CD4+ cells are the predominant lymphocyte subset. NO synthesis in hypersensitivity granulomas may therefore have multiple roles, including elimination of the source of antigen and the modulation of T-cell responses. Activated macrophages isolated from lesions induced by intracellular microorganisms or parasites have toxic effects on the offending pathogens, which are largely NO dependent [87]. Bacille Calmette–Guerin was one of
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the first agents shown to induce macrophage synthesis of nitrite and nitrate in mice [88], and Mycobacterium avium is one of the few inducing signals known for human macrophages [89]. Schistosoma will provoke NO synthesis in macrophages and endothelial cells [90, 91].

Granuloma cells produce cytokines which are known to regulate NO synthesis. The cytokine profiles vary with the maturity of the lesions. In most studies, pro-inflammatory cytokines, which would be anticipated to induce iNOS in macrophages, predominate in early lesions: IL-1β, TNF-α and IFN-γ [92]. In mature lesions, as elimination of the antigen is advancing, Th2 cytokines (IL-4 and IL-10) predominate [92] which would favour suppression of induced NO synthesis. Transforming growth factor-β has also been identified in granulomas [93]. In lung foreign body granulomas induced by dextran beads, IL-1α and iNOS have been shown to be induced in cells accumulating around the beads [94], but as yet the in vivo evidence for involvement of NO in immune granulomas is largely indirect. It was observed as long ago as 1967 that serum arginine levels were reduced in mice heavily infected with Schistosoma [95]. Mycobacterium, another infective agent which induces cytokine production during development, but decline in advanced lesions (S. N. Waddington, personal communication and Fig. 3). These experimental data in rodents support a role for NO in the pathogenesis of immune granulomas. Evidence of involvement in human disease is currently lacking.

Transplantation

There are now many reports of increased NO production during acute allograft rejection. Elevated serum nitrate levels have been found after rat small bowel [99], cardiac [100] and bone marrow [101] transplantation, and in the sponge matrix allograft model in mice [102] and rats [103]. There is also evidence that this occurs in humans rejecting bone marrow transplants [104] and, in preliminary reports, kidney transplants [105, 106]. This increased synthesis does not follow transplantation of isografts or syngeneic grafts. It can precede clinical symptoms by several days [104]. Urinary nitrite/nitrate is also elevated although in renal allografts interpretation of urinary nitrite/nitrate is complicated by changes in renal function, and levels may be reduced with acute renal failure due to cyclosporin toxicity, despite elevated serum levels [106]. Increased NO synthesis has also been monitored by EPR spectroscopy [99, 100].

The inducible isoform of NOS is responsible for the NO generation. This has been shown by detection of iNOS mRNA by RT–PCR [100] or Northern blot [107] and by immunohistochemistry, using iNOS-specific antibodies [100, 107, 108]. These studies show in cardiac and renal allografts that the iNOS is predominantly localized in infiltrating mononuclear cells, almost certainly macrophages. This is supported by evidence of nitrite production in the adherent macrophage fraction of the leucocyte population in sponge matrix allografts [103]. However, as some murine T-cell clones can synthesize NO [27], the exact contribution of T-cells is uncertain.

It is too early to be certain of the role that NO plays in the rejection process, or whether it is only relevant to acute cellular rejection. There are now several studies testing the effects of NO inhibition in the allograft reaction in vivo, but results are conflicting. Aminoguanidine, a relatively selective inhibitor of iNOS, has been shown to prolong rat cardiac [100] and lung [109] graft survival, and a small increase in survival was found with L-NMMA [110]. However, this compound caused weight loss and decreased survival in a mouse bone marrow transplantation model. The mechanism was uncertain, but was not apparently due to exacerbation of graft-versus-host disease histologically. There is a preliminary report that L-NAME causes exacerbation of renal allograft rejection [111].

CONCLUSION

There is now considerable evidence for the involvement of NO in immune-mediated diseases. Increased synthesis of NO has been demonstrated in a wide range of animal models and also in several
human diseases including arthritis, asthma, ulcerative colitis and allograft rejection. In most of these the source of the increased NO synthesis can be shown to be iNOS induced by pro-inflammatory cytokines. Humans also have an iNOS both in parenchymal cells and in macrophages, and upregulation of iNOS has been shown in arthritis, multiple sclerosis, ulcerative colitis and asthma. Although there is firm evidence both for the synthesis and source of NO in these diseases, elucidation of its role is more difficult because of the lack of selective inhibitors of the various isoforms of NOS. Glucocorticoids are effective inhibitors of the induction of iNOS and it is likely that some of their effects in inflammation may be due to reduction in NO synthesis. In general, it appears that NO may have both pro- and anti-inflammatory effects and that the balance between these may be determined by the timing of its synthesis during the inflammatory response, by the presence of other mediators such as superoxide and by the redox state of the tissue. In most experimental models the evidence is strongest for a role for NO in the effector phase of the immune response and, while there are good in vitro data showing that NO may affect the generation of the immune response, the evidence for such a role in models of disease is less convincing. It is clear that over the next few years our knowledge of the actions of NO in immune-mediated diseases will increase and may lead to new therapeutic strategies for these common disorders.

REFERENCES

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